

Supplementary Information

Design of small molecule-responsive microRNAs based on structural requirements for Drosha processing

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Supplementary methods

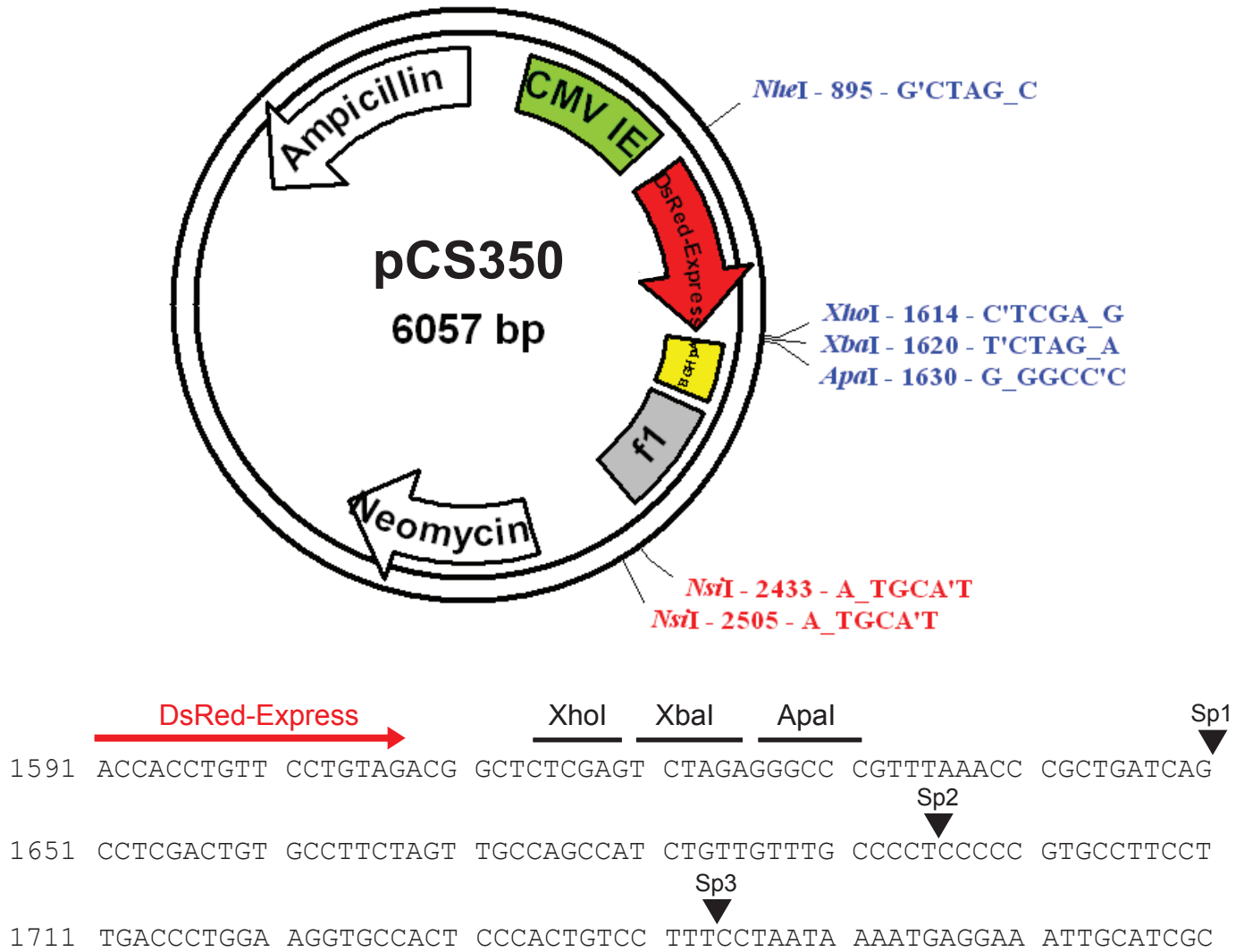


Figure S1 Plasmid map of pcDNA3.1(+) expressing DsRed-Express and sequence of the miRNA insertion site. The gene encoding DsRed-Express was cloned into *NheI*/*XhoI* with a Kozak consensus sequence (CGCCACC) immediately upstream of the start codon. DsRed-Express is expressed from the constitutive and strong CMV IE promoter. MiRNAs were cloned into *XbaI*/*ApaI* located in the DsRed-Express 3' untranslated region, where the resulting miRNA sequences are listed in Supplementary Table 1. Multiple copies of miRNAs were sequentially inserted by amplifying the miRNA with the PCR primers listed in Experimental Procedures and cloned into *XhoI*/*XbaI*. Sp1, Sp2, and Sp3 mark the 3' end of miRNAs with an intervening sequence of 20, 64, and 112 nt, respectively. The 3' end of the DsRed-Express coding region is designated in red. Numbering corresponds to the original pcDNA3.1(+) vector.

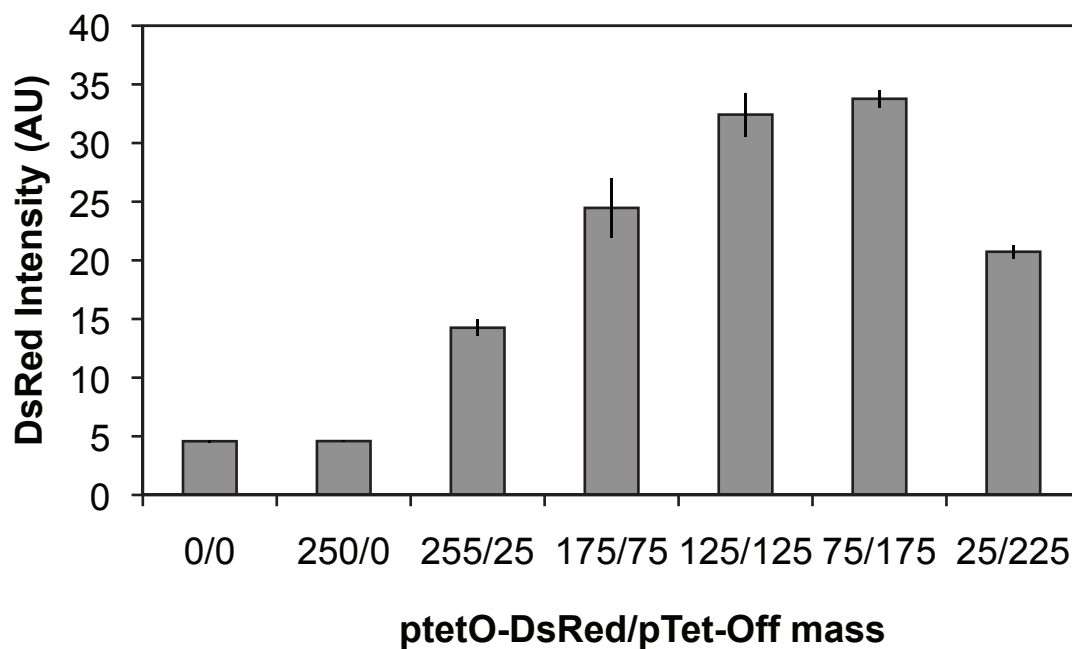
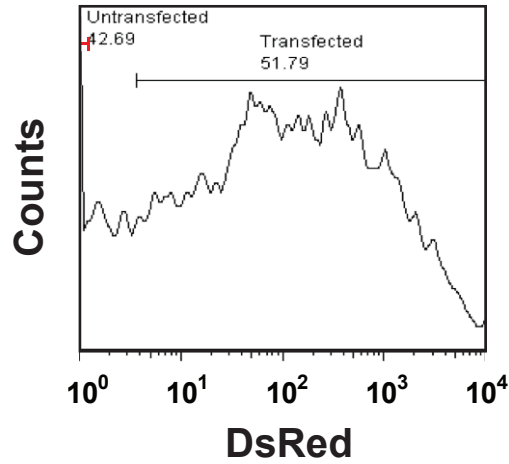
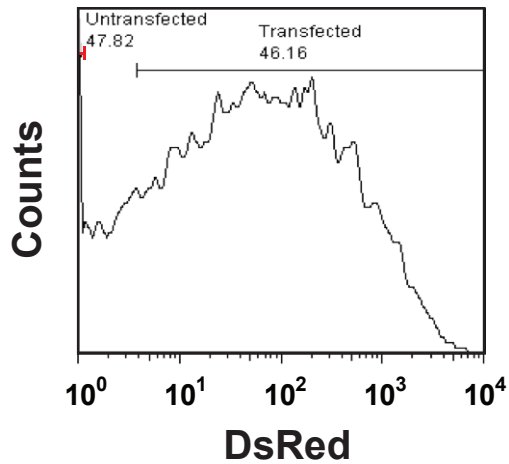


Figure S2 Optimization of the transfected plasmid ratio for the tet inducible promoter system. Different amounts of plasmids ptetO-DsRed and pTet-Off were cotransfected into 293 d2EGFP cells in the absence of doxycycline. A total of 250 ng of plasmid was maintained for each cotransfection. Three days post-transfection, DsRed levels were measured by flow cytometry. Each reported DsRed intensity reflects the geometric mean of the fluorescence distribution. Error bars reflect the range of values from three independent transfections conducted in the same cell-culture plate.

No
miRNA



th1 (1X)



th1 (4X)

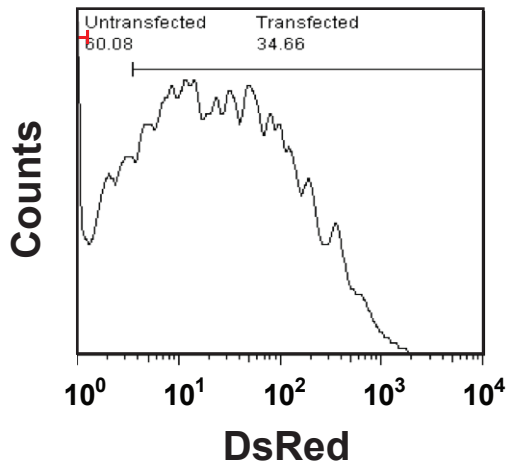


Figure S3 Gating for transfected and untransfected cells using DsRed fluorescence. GFP-positive cells with detectable and undetectable DsRed fluorescence (FL3) were gated as transfected (black bars) and untransfected (red bars) cells, respectively. Representative histograms from flow cytometry analysis are shown for the transient transfection of constructs lacking any miRNAs (No miRNA) or containing one copy (1X) or four copies (4X) of a theophylline-responsive miRNA (th1). DsRed fluorescence was readily detectable even for four miRNA copies in the 3' untranslated region of the *dsRed* gene.

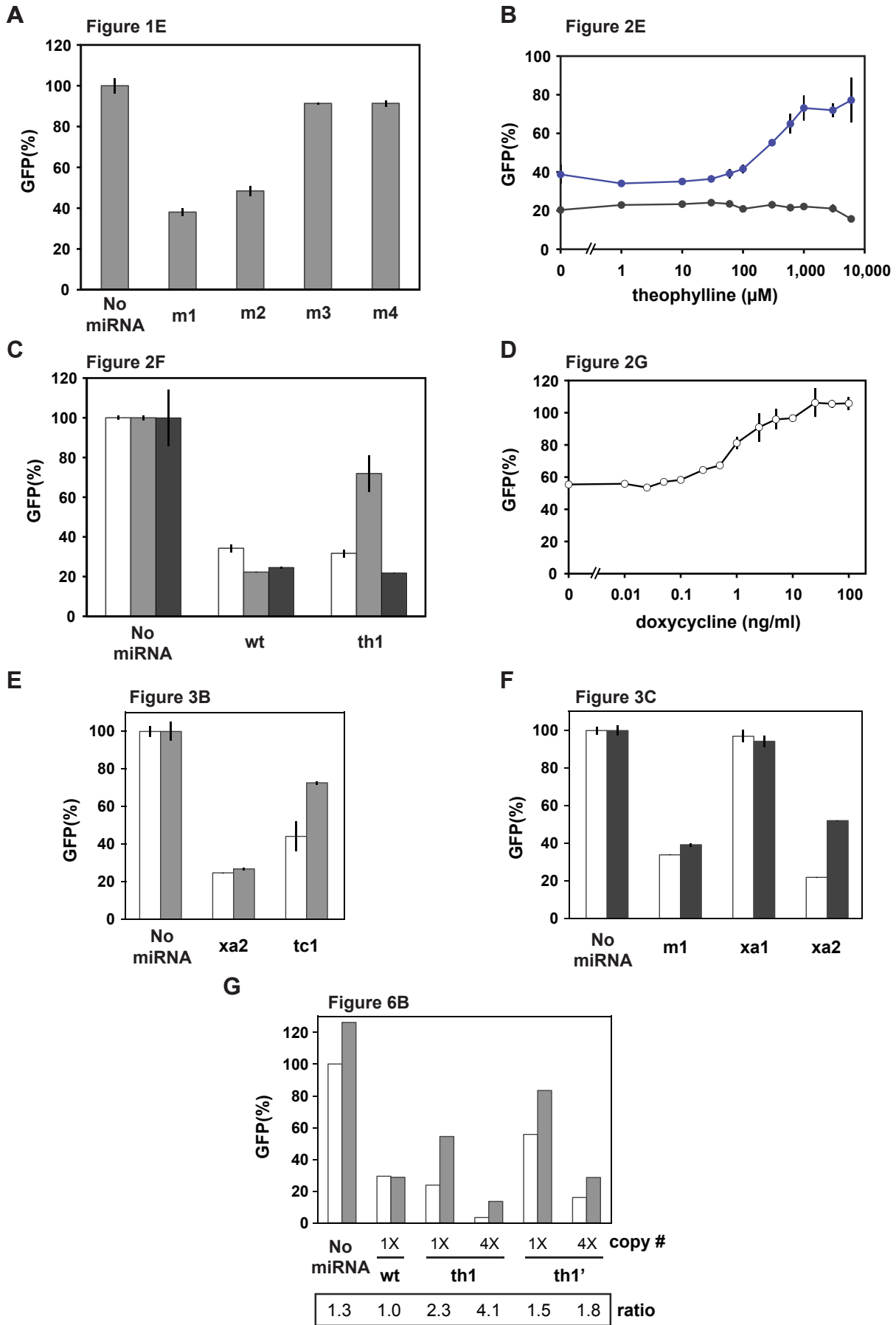


Figure S4 See next page for figure legend.

Figure S4 Cell-culture assay results are similar across repeated independent experiments. Data from experiments conducted as described in (A) Figure 1E, (B) Figure 2E, (C) Figure 2F, (D) Figure 2G, (E) Figure 3B, (F) Figure 3C, and (G) Figure 6B are shown. Error bars are described in each figure.

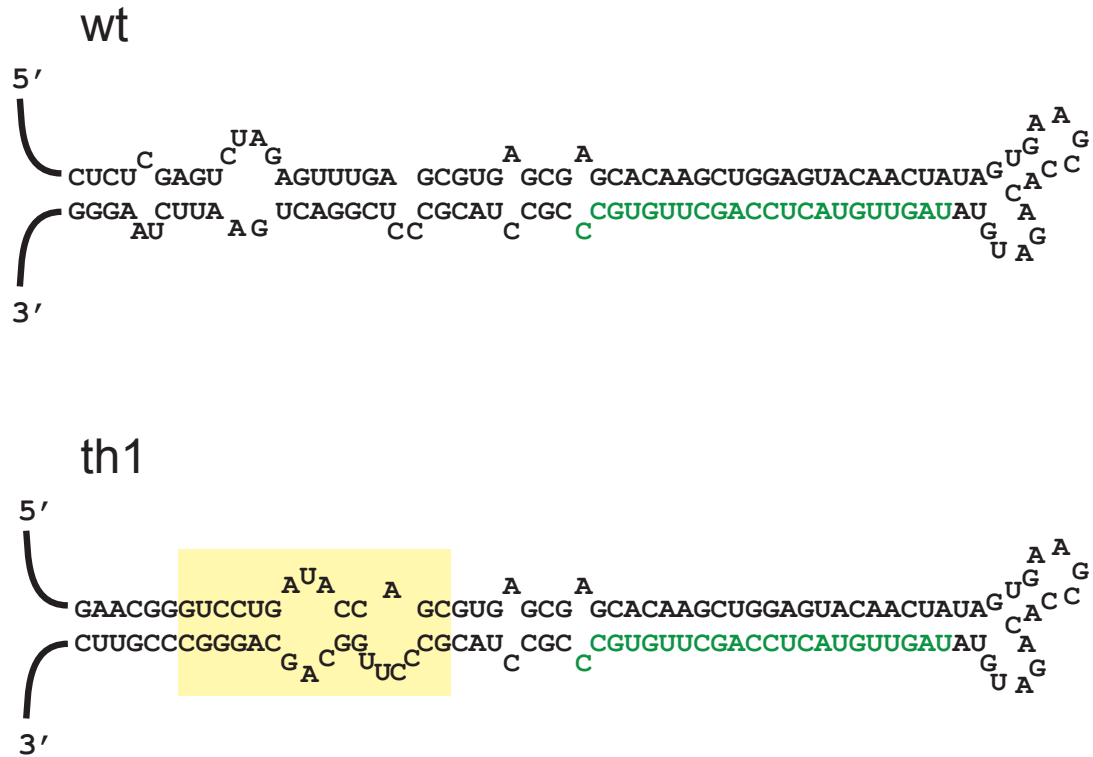
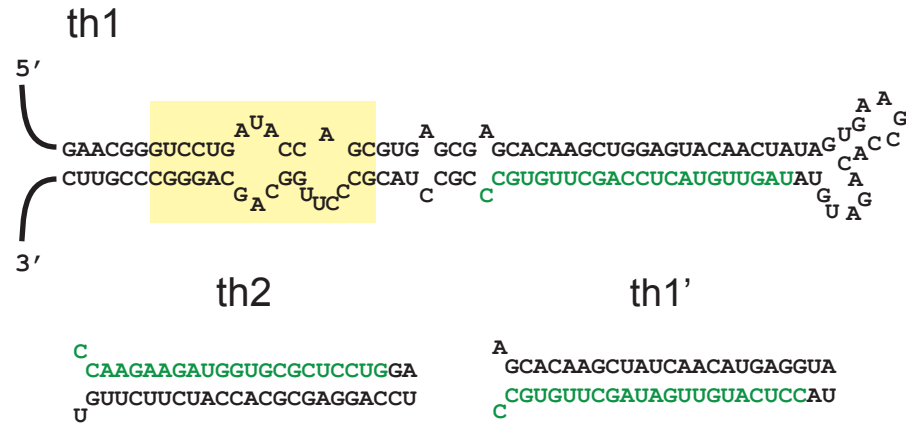


Figure S5 Sequence and secondary structure of miRNAs targeting GFP. Basal segments contain sequences that are similar to miR-30a (wt) or the theophylline aptamer (th1). The aptamer insertion site is indicated by the yellow box according to Figure 3A and the mature miRNA sequence complementary to the GFP transcript is indicated in green text.

A



B

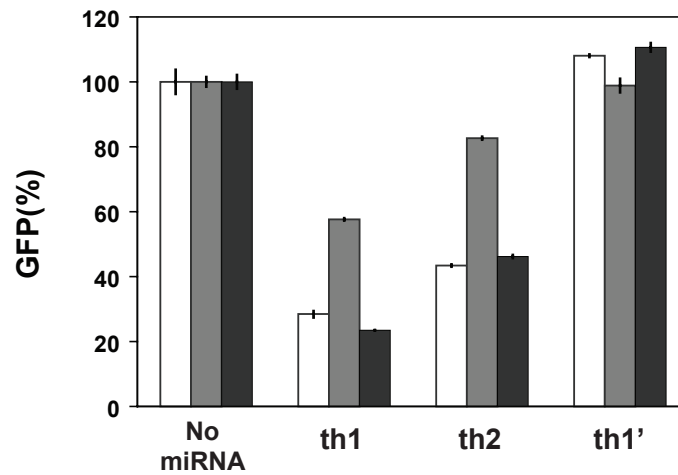


Figure S6 Ligand-responsive miRNAs can accommodate different mature miRNA sequences to tailor the gene silencing output of the regulatory system. (A) The mature miRNA sequence contained within the upper stem of th1 was modified to target a different sequence within the GFP mRNA (th2) or abolish targeting (th1'). All miRNAs contain the theophylline aptamer in the basal segments. The aptamer insertion site is indicated by the yellow box according to Figure 3A, and mature miRNA sequences are indicated in green text. The GFP-targeting miRNAs were cloned into the plasmid constructs and characterized through the cell-culture assay described in Figure 1D,E. (B) GFP silencing results for theophylline-responsive miRNA constructs (th1, th2, or th1') transiently transfected in the absence (white) or presence of either 5 mM theophylline (gray) or 1 mM caffeine (black). See Materials and Methods for a description of data normalization. Error bars reflect the range of values from two independent transfections conducted in the same cell-culture plate.

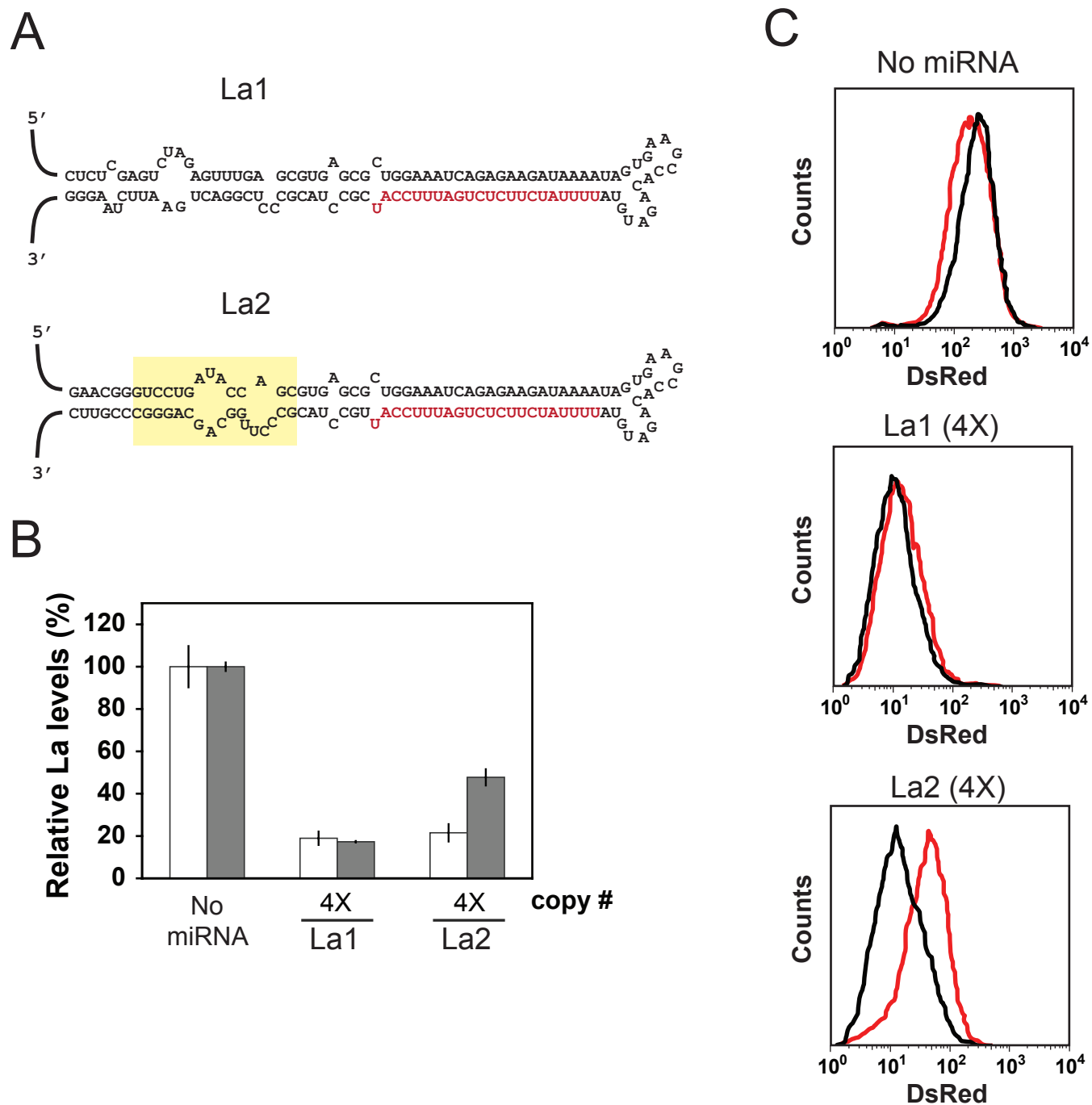


Figure S7 See next page for figure legend.

Figure S7 Ligand-responsive miRNA clusters can effectively control expression of endogenous gene targets. (A) Sequence and secondary structures of miRNAs that target the endogenous La gene. Color schemes are identical to Figure 1B, except that the mature miRNA sequence complementary to the La transcript is indicated in red. Sequences similar to miR-30a (La1) or the theophylline aptamer (La2) were inserted into the miRNA basal segments. miRNAs were cloned into the plasmid constructs described in Figure 1D at the indicated copy numbers using the largest spacer length tested (112 nt). The resulting constructs were stably transfected into HEK 293-Flp-In cells. (B) Relative La transcript levels for stable cell lines expressing the La-targeting miRNA constructs in the presence (gray) or absence (white) of 1.5 mM theophylline. La transcript levels were measured through qRT-PCR and normalized to GAPDH encoding transcript levels as an internal control. Relative levels are normalized to that of cells stably transfected with the construct lacking a miRNA (No miRNA) grown under the same conditions. Error bars represent the calculated error of quadruplicate qRT-PCR wells of each sample. (C) Flow cytometry histograms for DsRed levels from the La-targeting miRNAs. The stable cell lines tested in B were grown in the presence (red) or absence (black) of 1.5 mM theophylline for over a week prior to flow cytometry analysis. Histograms are representative of two independent experiments.

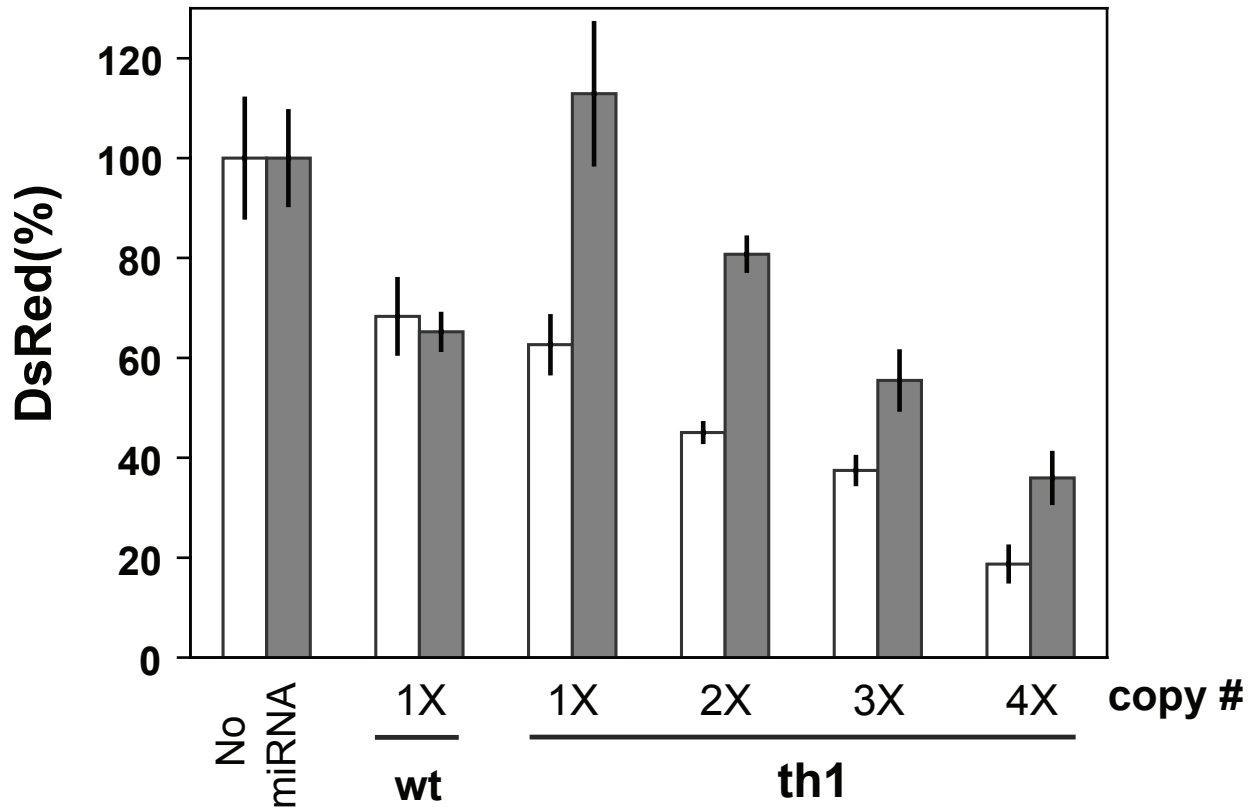


Figure S8 Ligand-responsive miRNAs control gene expression in cis through transcript destabilization. Multiple copies (#X) of the GFP-targeting theophylline-responsive miRNAs (th1) were cloned into the plasmid constructs described in Figure 1D using the largest spacer length tested (112 nt). wt was used as a negative control to allow direct comparison to Figure 4B. 293 cells stably expressing GFP were transiently transfected with the resulting constructs in the presence (gray) or absence (white) of 5 mM theophylline. DsRed transcript levels were measured through qRT-PCR and normalized to transcript levels of the Neomycin resistance gene expressed from the same transfected plasmid. Relative levels were normalized to that of cells transfected with the construct lacking a miRNA (No miRNA) grown under the same conditions. Error bars represent the calculated error of quadruplicate qRT-PCR wells for each sample.

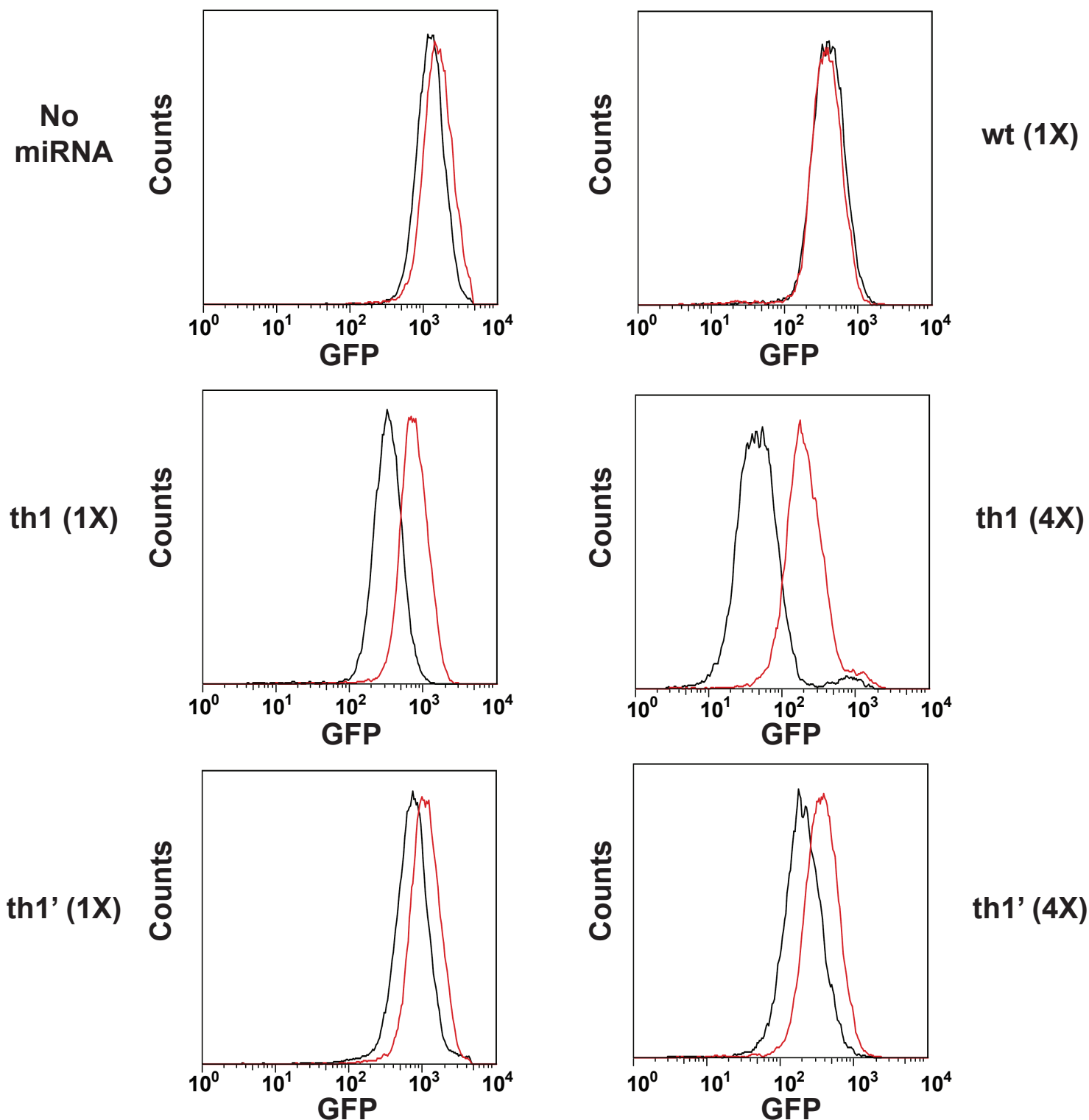


Figure S9 Flow cytometry histograms for 293-Flp-In cells stably expressing the self-targeting miRNA constructs. miRNAs were located in the 3' UTR of the trans-targeted transcript encoding GFP. Constructs lacking any miRNAs (No miRNA), one copy of a self-targeting miRNA with basal segments containing sequences similar to miR-30a (wt), one (1X) or four (4X) copies of a theophylline-responsive self-targeting miRNA (th1), and one or four copies of a theophylline-responsive non-targeting miRNA (th1') were characterized, where multiple copies were separated by the largest spacer length tested (112 nt). Stable cell lines were grown for over one week in the presence (red) or absence (black) of 1.5 mM theophylline prior to flow cytometry analysis.

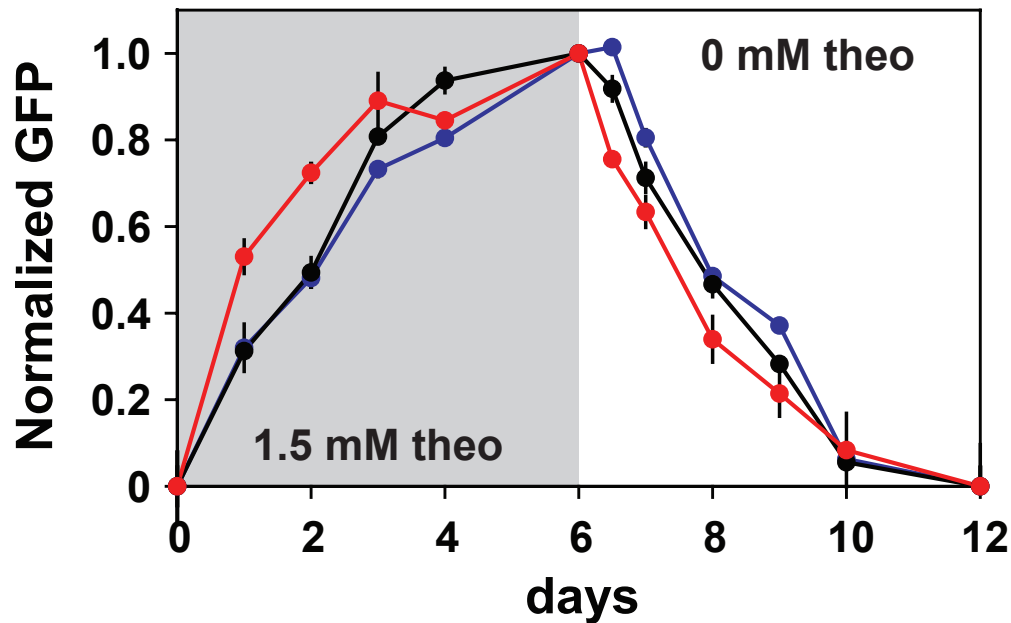


Figure S10 Dynamics of ligand-responsive miRNA regulation in response to a step-change in ligand concentration. Data are shown for cells expressing the miRNA constructs containing four copies of th1' (red), one copy of th1 (blue), or four copies of th1 (black). Cells were grown in the presence of 1.5 mM theophylline for six days then transferred to media without theophylline for six days. Time course data were rescaled to fall between zero when theophylline was added at the beginning of the time course and one when theophylline was removed in the middle of the time course to compare the dynamics of the approach to steady-state between ligand-responsive miRNAs acting through different regulatory mechanisms. Error bars reflect the range of values from cells grown in two separate wells in the same cell-culture plate.

Table S1. Sequences for ligand-responsive and control miRNAs. Each sequence is written 5' to 3' and represents the final construct cloned into XbaI and ApaI within pcDNA3.1(+). th3, th1 Sp1, th1 Sp2, and th1 Sp3 represent a second copy of th1 cloned into XhoI and XbaI in pcDNA3.1(+) already containing th1 to test the efficacy of two miRNAs separated by different spacer sequences. Color codes: gray, restriction sites; blue, aptamer; green, designed guide strand sequence. The database # is included for plasmid requests.

Name	Sequence	Aptamer	Database #
wt	TCTAGAGTTTGTACAGTGAGCGAGCACAAGCTGGAGTACAACATATAGTGAAGCCA CAGATGTATAGTTGTACTCCAGCTTGTGCCGTCCTACTGCCTCGGACTGAATTC ATAGGGCCC		pCS351
m1	TCTAGAACGGGAAGTAATTACAGTGAGCGAGCACAAGCTGGAGTACAACATATAG TGAAGCCACAGATGTATAGTTGTACTCCAGCTTGTGCCGTCCTACTGCCACATA GGGCCC		pCS1246
m2	TCTAGAACGGGAAACACAGTGAGCGAGCACAAGCTGGAGTACAACATATAGTGAA GCCACAGATGTATAGTTGTACTCCAGCTTGTGCCGTCCTACTGCCTCGGGCCC	None	pCS1215
m3	TCTAGAACGGGAAACACAGTGAGCGAGCACAAGCTGGAGTACAACATATAGTGAA GCCACAGATGTATAGTTGTACTCCAGCTTGTGCCGTCCTACTGCCGGGCCC		pCS1241
m4	TCTAGAACGGGAAACACAGTGAGCGAGCACAAGCTGGAGTACAACATATAGTGAA GCCACAGATGTATAGTTGTACTCCAGCTTGTGCCGTCCTACTGGGGCCC		pCS1242
th1	TCTAGAACGGGTCCGTGATACCAGCGTGAGCGAGCACAAGCTGGAGTACAACATAT AGTGAAGCCACAGATGTATAGTTGTACTCCAGCTTGTGCCGTCCTACGCCCTTG GCAGCAGGGCCC		pCS1183
th1'	TCTAGAACGGGTCCGTGATACCAGCGTGAGCGAGCACAAGCTATCAACATGAGGT AGTGAAGCCACAGATGTATCCTCATGTTGATAGCTTGTGCCGTCCTACGCCCTTG GCAGCAGGGCCC		pCS1258
th2	TCTAGAACGGGTCCGTGATACCAGCGTGAGCGCCCAAGAAGATGGTGCGCTCCTGG AGTGAAGCCACAGATGTCCAGGAGCGCACCATCTTCTTGTGCGCTACGCCCTTG GCAGCAGGGCCC	Theophylline	pCS1664
th3	TCTAGACGCCAGAAATGATACCAGCGTGAGCGAGCACAAGCTGGAGTACAACATAT AGTGAAGCCACAGATGTATAGTTGTACTCCAGCTTGTGCCGTCCTACGCCCTTG GCAGCATTTCTGGCGCCTAGAACGGGTCCGTGATACCAGCGTGAGCGAGCACAAGC TGGAGTACAACATATAGTGAAGCCACAGATGTATAGTTGTACTCCAGCTTGTGCC CGCCTACGCCCTTGGCAGCAGGGCCC		pCS1229
th1 Sp1	TCTAGAACGGGTCCGTGATACCAGCGTGAGCGAGCACAAGCTGGAGTACAACATAT AGTGAAGCCACAGATGTATAGTTGTACTCCAGCTTGTGCCGTCCTACGCCCTTG GCAGCAGGGCCCCGTTTAAACCCGCTGATCAGCCTAGAACGGGTCCGTGATACCAG CGTGAGCGAGCACAAGCTGGAGTACAACATATAGTGAAGCCACAGATGTATAGTT GTACTCCAGCTTGTGCCGTCCTACGCCCTTGGCAGCAGGGCCC		pCS1321
th1 Sp2	TCTAGAACGGGTCCGTGTATTACCAGTGAGCGAGCACAAGCTGGAGTACAACATATA GTGAAGCCACAGATGTATAGTTGTACTCCAGCTTGTGCCGTCCTAGGTGACGG GCCCCGTTTAAACCCGCTGATCAGCCTCGACTGTGCCTTCTAGTTGCCAGCCATC TGTTGTTTGGCCCTCCTAGAACGGGTCCGTGATACCAGCGTGAGCGAGCACAAGC TGGAGTACAACATATAGTGAAGCCACAGATGTATAGTTGTACTCCAGCTTGTGCC CGCCTACGCCCTTGGCAGCAGGGCCC	Theophylline	pCS1322
th1 Sp3	TCTAGAACGGGTCCGAGGTCGACGTGAGCGAGCACAAGCTGGAGTACAACATATA GTGAAGCCACAGATGTATAGTTGTACTCCAGCTTGTGCCGTCCTACGTGTATTA CCCAGGGCCCCGTTTAAACCCGCTGATCAGCCTCGACTGTGCCTTCTAGTTGCCA GCCATCTGTTGTTTGGCCCTCCCCCGTGCCCTTCCTTGACCCTGGAAGGTGCCAC TCCCCTGTCTTTTCTAGAACGGGTCCGTGATACCAGCGTGAGCGAGCACAAGC TGGAGTACAACATATAGTGAAGCCACAGATGTATAGTTGTACTCCAGCTTGTGCC CGCCTACGCCCTTGGCAGCAGGGCCC		pCS1323

Supplementary Table 1 cont'd.

Name	Sequence	Aptamer	Database #
tc1	TCTAGAACGGGTCC CTAAACATACC GTGAGCGAGCACAAAGCTGGAGTACAACCTA TAGTGAAGCCACAGATGTAT AGTTGTACTCCAGCTTGTGCC TGCCTAC GGAGAG GTGAAGAATACGACCACCTAG GGCCC	Tetracycline	pCS1217
xa1	TCTAGAACGGGTCC GTGTATTACC TGAGCGAGCACAAAGCTGGAGTACAACCTATA GTGAAGCCACAGATGTAT AGTTGTACTCCAGCTTGTGCC CGCCTA GGTCGACGG GCCC	Xanthine	pCS1218
xa2	TCTAGAACGGGTCC CGAGGTCGAC GTGAGCGAGCACAAAGCTGGAGTACAACCTATA GTGAAGCCACAGATGTAT AGTTGTACTCCAGCTTGTGCC CGCCTAC GTGTATTAC CCCAG GGCCC		pCS1244
La1	TCTAGAGTTTGACAGTGAGCGCTGGAAATCAGTGAAGATAAAATAGTGAAGCCA CAGATGTAT TTTTATCTTCACTGATTTCAT TGCCTACTGCCTCGGACT GAATTC ATAGGGCCC	None	pCS1676
La2	TCTAGAACGGGTCC TGATACCAGC GTGAGCGCTGGAAATCAGTGAAGATAAAAT AGTGAAGCCACAGATGTAT TTTTATCTTCACTGATTTCAT CGCCTAC GCCCTTG GCAGCA GGGCCC	Theophylline	pCS1677

Supplementary Methods

qRT-PCR. The following oligos were used for qRT-PCR. La protein (Acc # X13697): La_fwd, 5'-GGTTGAACCGTCTAACAACAG-3'; La_rev, 5'-ATGTCATCAAGAGTTGCATCAG-3'; GAPDH (Acc # NM_002046): GAPDH_fwd, 5'-GAAGGTGAAGGTCGGAGTC-3'; GAPDH_rev, 5'-GAAGATGGTGATGGGATTTC-3'; DsRed-Express: DsRed_fwd, 5'-AAGAAGACTATGGGCTGGGA-3'; DsRed_rev 5'-CGATGGTGTTAGTCCTCGTTG-3'; and the Neomycin resistance gene: NeoR_fwd, 5'-ACCTTGCTCCTGCCGAGAAAGTAT-3'; NeoR_rev, 5'-ATGTTTCGCTTGGTGGTCGAATGG-3'. Transcript levels were measured by qRT-PCR under either transient or stable transfection conditions. For transient transfections 293 cells were washed with 1X PBS three days post-transfection and total RNA was extracted using the RNeasy Mini kit (Qiagen) according to the manufacturer's instructions. For stable transfections, cell lines were grown for over a week in the presence or absence of 1.5 mM theophylline prior to total RNA extraction. Total RNA samples were treated with DNase I at 37°C for 20 minutes and purified using a NucAway column (Ambion). Up to 5 µg of purified RNA was reverse-transcribed using Superscript III reverse transcriptase (Invitrogen) according to the manufacturer's instructions using the reverse primers for each pair of gene target and loading control (DsRed/NeoR, La/GAPDH) followed by the recommended incubation with RNase H. qRT-PCR was conducted with the resulting cDNA on the iCycler iQ system (BioRAD) according to the manufacturer's instructions. Samples were prepared in quadruplicate using the iQ SYBR green supermix and data were analyzed using the iCycler iQ software. The mean of the resulting C_T values for the target gene of each sample were subtracted from the mean C_T value for the control gene. The resulting values were then converted from log₂ to linear scale and normalized to the value for the sample lacking any miRNA transfected with the same

concentration of ligand. The reported sample error was calculated using the following expression:

$$\text{Sample error} = \frac{2^{\text{AVE}(\text{Cont}) - \text{AVE}(\text{Target}) - \frac{1}{2}[\text{SD}(\text{Cont}) - \text{SD}(\text{Target})]} - 2^{\text{AVE}(\text{Cont}) - \text{AVE}(\text{Target})}}{[2^{\text{AVE}(\text{Cont}) - \text{AVE}(\text{Target})}]_{\text{Neg}}}$$

where AVE and SD are the respective average and standard deviation of each quadruplicate sample, Cont and Target are the loading control and target, respectively, and Neg is the sample lacking a miRNA transfected with the same ligand concentration as the sample in question.

Design of mature miRNAs. The mature miRNA sequences targeting GFP and La were selected to be completely complementary to a single site within each coding region. GFP was targeted at positions 416 (GGCACAAGCTGGAGTACAATA) and 281 (TCCAGGAGCGCACCATCTTCTT), and La was targeted at position 310 (ATGGAAATCAGTGAAGATAAAA). The first GFP-targeting sequence (416) was derived from the OpenBiosystems shRNA eGFP Positive PSM2 vector control, whereas the second GFP-targeting sequence (281) and the La-targeting sequence (310) were generated using Dharmacon's online siRNA design software. Mature miRNA sequences were then introduced into the top or bottom of the upper stem of the base miRNA.